Transforming growth factor β production during rat cytomegalovirus infection

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We analysed the production of transforming growth factor β (TGF-β) during a cytomegalovirus (CMV) infection in a rat model system. Splenocytes from immunocompetent rats infected with rat CMV (RCMV) released increased amounts of TGF-β. TGF-β production was also evident in RCMV-infected radiation-immunosuppressed rats; their sera inhibited the interleukin 2-induced proliferation of T cells, which could be restored by anti-TGF-β antibodies. In addition, TGF-β production could be visualized immunohistologically in the lungs, spleen, liver and bone marrow of radiation-immunosuppressed infected rats. The virus directly induced this cytokine since TGF-β was produced upon RCMV infection in vitro. The induction of TGF-β production may contribute to immunosuppression during CMV infection.

Introduction

Cytomegalovirus (CMV) infections cause immunosuppression, characterized by depression of peripheral blood lymphocyte blastogenesis and of interferon γ (IFN-γ) production in vitro in response to mitogens and CMV antigen (Rinaldo et al., 1980). In cultures of peripheral blood lymphocytes, CMV infection also leads to reduced proliferative responses. However, only small numbers of lymphocytes are found infected, which mostly express only the immediate early responses. However, only small numbers of lymphocytes are found infected, which mostly express only the immediate early antigen (Rice et al., 1984). Infection of monocytes, on the other hand, has been shown to mediate inhibition of lymphocyte responses to mitogens (Carney et al., 1981). This leads to diminished antimicrobial resistance, particularly conspicuous in persons with pre-existing immunodeficiencies. In transplant patients, CMV infection is therefore often followed by serious bacterial or fungal diseases (Ho, 1982).

Lymphocyte proliferation is regulated by several pro-inflammatory cytokines including interleukin 1 (IL-1), IL-6, tumour necrosis factor α (TNF-α) and transforming growth factor β (TGF-β) (Dinarello, 1989; Vink et al., 1990; Wahl et al., 1988). Of these, IL-1 induces T cell proliferation whereas TGF-β and the recently described IL-1 receptor antagonist protein (IRAP) have strong inhibitory activity (Wahl et al., 1988; Eisenberg et al., 1990). CMV infection of monocytes induces the expression of IL-1 (Dudding et al., 1989), but abrogation of IL-1 activity by infected monocytes has also been reported (Dudding & Garnett, 1987). It is not clear which cytokines dominate during CMV infection in vivo.

We analysed the production of TGF-β after experimental CMV infection in vitro and in vivo. Rat CMV (RCMV) very much resembles its human counterpart: it replicates only poorly in the immunocompetent host, but reaches high titres in immunosuppressed animals (Stals et al., 1990). RCMV also induces immunosuppression, as evidenced by a reduced antibody response to sheep red blood cells in immunocompetent rats and by inhibition of haematopoietic recovery in whole body-irradiated rats (Stals et al., 1990; Bruggeman et al., 1985). We demonstrate the expression of TGF-β during RCMV infection and suggest that it plays an important role in CMV pathogenesis.

Methods

- Virus. Stocks of RCMV were prepared as 10% (w/v) homogenates of salivary gland tissue taken from brown Norway rats 3 weeks p.i. with 10⁷ p.f.u. of RCMV via the intraperitoneal route. RCMV was passaged in rat embryo fibroblasts. When CPE was at a maximum, supernatants were harvested and centrifuged at 900 g for 10 min. Virus was plaque-titrated and stored in aliquots at −70 °C until use.

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Animals. Inbred specific pathogen-free male brown Norway rats were obtained from Harlan. The animals were kept in filter top cages. The experimental protocols were approved by the institutional Animal Welfare Committee.

Experimental design. Immunocompetent and whole-body-irradiated (5 Gy at 350 cGy/min at day –1) rats were infected via the intraperitoneal route with 10^7 p.f.u. of RCMV on day 0. Blood samples obtained after orbital puncture were allowed to clot at 4 °C for 1 h, centrifuged and kept at –20 °C until use. At indicated times p.i., erythrocyte-depleted cell suspensions were prepared from the spleens of immunocompetent rats. Splenocytes were cultured in 24-well plates at a density of 2 x 10^8 cells per well in serum-free Opti-MEM medium (Gibco) containing 2 mM t-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin for 48 h at 37 °C in a 5% CO2 atmosphere (conditioned media). Concanavalin A (Con A) was added at a concentration of 5 µg/ml and the supernatants were stored at –20 °C until use. Splenocytes were cultured in 96-well flat-bottomed plates at a density of 10^6 per ml and after 72 h incubation the cultures were pulsed with 1 µCi [H]thymidine (sp. act. 0.7–1.1 x 10^6 MBq/mmol; Amersham) for 16 h. Cells were harvested on glass filters and the incorporated radioactivity was measured in a Betaplate scintillation counter (LKB-Wallac).

IFN-γ assay. IFN-γ levels were determined using a rat IFN-γ-specific ELISA (van der Meide et al., 1989). Briefly, wells of a 96-well microtitre plate (Flow Laboratories) were coated for 16 h at 4 °C with 100 µl of a PBS solution containing DB-1 monoclonal antibody (MAb) at 10 µg/ml. The wells were washed 10 times with PBS containing 0.05% Tween 20 (wash buffer). After blocking with 200 µl of a 2% BSA solution in PBS, the plates were emptied and incubated with the samples. As a positive control, recombinant rat IFN-γ was used. Thereafter the wells were extensively washed and refilled with 100 µl of biotinylated antibody DB-12 at 2 µg/ml in PBS and incubated. After 10 wash cycles, 100 µl of anti-biotin–peroxidase conjugate (1 µg/ml diluted in 1% BSA) was added to each well. Again the plate was incubated and subsequently washed 10 times. Then 100 µl of a solution of the substrate tetramethyl benzidine (Sigma) was added to each well. The substrate conversion was stopped by the addition of 30 µl 6 M H2SO4 and the absorbance was read at 450 nm. All incubations were for 1 h at 37 °C.

IL-1 assay. IL-1 bioactivity was measured using the D10 bioassay (Hopkins & Humphreys, 1989; Miltenburg et al., 1992). Briefly, 10^4 cells were incubated in flat-bottomed well plates in the presence of Con A (3 µg/ml) and 10 U/ml recombinant IL-2 with the samples to be tested and 1 µCi [H]thymidine (Amersham) was added between 64 and 68 h later. IL-1 concentrations were calculated from a standard curve obtained with recombinant IL-1α (Genzyme), showing the relationship between incorporation and cytokine concentration. The D10 assay is highly sensitive (detection limit 0.1 U/ml) and specific (no response to TNF-α or IL-8) under these conditions.

IL-2 assay. A cloned murine T cell line (CTLL-16) was used to determine immunosuppressive activity in sera (Gillis et al., 1978). Briefly, 3 x 10^4 cells were incubated in 96-well plates with samples to be tested in the presence of 10 U/ml IL-2. Sera were used at a 1:20 dilution and preincubated with neutralizing antibodies against TGF-β1 and TGF-β2 (R and D Systems) or pre-immune rabbit serum for 30 min at 37 °C. After 24 h cells were labelled with 1 µCi/ml [H]thymidine for 16 h.

TGF-β assay. TGF-β activity in tissue culture supernatant was determined in a biological assay using ML-CCL64 mink lung epithelial cells (Danielpour et al., 1989). The cells were collected during their logarithmic growth phase and suspended at a concentration of 6 x 10^4 per ml in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) containing 0.2% foetal calf serum (FCS). Aliquots (50 µl) of the suspension were distributed in flat-bottomed 96-well plates (Nunc) and incubated at 37 °C for 5 h. Native or heat-activated (5 min, 80 °C) samples were then added to the wells at various dilutions in the presence or absence of neutralizing rabbit anti-TGF-β1 or -TGF-β2 antisera. After 20 h the cells were pulsed with 1 µCi [H]thymidine (Amersham) for 4 h and the incorporated radioactivity was counted. The cytokine concentrations were determined from a standard curve which was established with known amounts of recombinant TGF-β2. The data represent the mean values ± SEM of [H]thymidine uptake of triplicate cultures.

Histology. Organ samples were fixed in paraformaldehyde–lysine periodate, decalcified (bone marrow) and embedded in paraffin. Paraffin sections (3 μm) were deparaffinized and rehydrated in TBS (0.05 M Tris–HCl pH 7.6) which was used for all washings. The sections were incubated with 1% H2O2 in methanol for 15 min to block endogenous peroxidase activity and preincubated for 20 min at room temperature with normal goat serum (diluted 1:40) to reduce non-specific binding. Sections were examined for the presence of virus antigen as described previously (Stals et al., 1990). Briefly, the slides were washed and incubated overnight at 4 °C with MAbs (8 or 35) directed against a 48 kDa nuclear and 29 kDa cytoplasmic RCMV late antigen (Brunet et al., 1987; Meijer et al., 1986), respectively. All reagents were diluted in PBS containing 0.1% BSA and titrated to obtain optimum results. Subsequently, slides were washed three times for 5 min and incubated for 30 min at room temperature with the diluted conjugates (peroxidase-labelled rabbit anti-mouse antibody; Sigma) containing 1% normal rat serum. Thereafter, slides were washed and peroxidase activity was visualized using 0.003% H2O2 and 0.5% 3,3’-diaminobenzidine in 0.05 M Tris–HCl buffer pH 8.3. TGF-β immunostaining was performed as described previously (Teers & Dorrington, 1993). Briefly, sections were deparaffinized and endogenous peroxidase activity was blocked with 1% H2O2 in methanol for 30 min; sections were then washed with 0.01 M TBS pH 7.6 and incubated with 0.1 M glycine in TBS. After incubation with hyaluronidase (1 mg/ml, Sigma) in 0.1 M sodium acetate buffer pH 5.5 for 30 min at 37 °C, the slides were washed, blocked with 5% normal horse serum for 30 min and incubated overnight at 4 °C with antibodies against TGF-β1 (Flanders et al., 1988) or TGF-β2 (van den Eijnden-van Raaij et al., 1990) at different dilutions in TBS containing 1% BSA and 0.2% Tween 20. The slides were then rinsed and treated with a biotinylated horse anti-rabbit antibody (Vector Laboratories), washed again and incubated for at least 60 min with components A and B of the ABC staining kit (Vector Laboratories). Both components were diluted 1:250 and prepared at least 15 min before addition. Slides were washed and peroxidase activity was visualized as described above. For double labelling experiments, chicken antibodies against TGF-β1 (R and D Systems), a biotinylated rabbit anti-chicken antibody (Vector Laboratories) and a phosphatase-conjugated rabbit anti-mouse antibody (Sigma) were used. Peroxidase and phosphatase activity were subsequently visualized using 3-amino-9-ethylcarbazole and Fast BB salt (Sigma), respectively. All washes were in TBS; slides were counterstained with haematoxylin, dehydrated and mounted.

Cytokine induction. Rat fibroblast cultures were prepared from 17-day-old embryos in DMEM containing 2% FCS, penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml). Cells were grown in plastic culture flasks (Nunc) at 37 °C in a humidified CO2 incubator and used at the third passage. Cells (10^6 per well) were then seeded into 9-well plates (Nunc) and incubated until they had reached subconfluency. Cells were infected with RCMV for 1 h (m.o.i. 3) or...
treated with lipopolysaccharide (LPS, 1 µg/ml) and cultured for 2 days in DMEM supplemented with 1% BSA. RCMV was preincubated with normal or RCMV-neutralizing rat serum (1:20 dilution, 30 min, 37 °C). Virus preparations and culture media were found to contain < 60 pg/ml of contaminating endotoxin as tested by the COA-Test (Chromogenix). At 24 and 48 h after inoculation, culture fluid was collected, centrifuged and stored at −20 °C until use. Supernatants were exposed to UV irradiation before use in the bioassay.

Results

Detection of TGF-β in RCMV-infected immunocompetent rats

Immunocompetent rats that had been given 10⁶ p.f.u. of RCMV via the intraperitoneal route efficiently controlled the infection. Antigen-containing cells were found mainly in the spleen with peak numbers observed at day 5 p.i. (Fig. 1a). During acute RCMV infection, T cell activity is detectable around day 7 p.i. (Haagmans et al., 1994a); in the conditioned media of splenocytes obtained at different times p.i., IFN-γ activity was found on days 3 and 7 p.i. (63 and 20 U/ml, respectively). In vitro, a transient immunosuppression was evident which coincided with immunosuppression observed in vivo (Bruggeman et al., 1985), since Con A-induced IFN-γ secretion by spleen cell cultures was reduced at days 3 and 7 p.i. (Fig. 1b). Similarly, proliferative responses of spleen leukocytes to Con A were inhibited at day 7 p.i. but restored at day 12 p.i. (data not shown). However, it is questionable whether this in vitro approach truly reflects the same kind of immunosuppression that occurs in vivo. Production of TGF-β, a prototype immunosuppressive cytokine, may be more relevant for immunosuppression in vivo. The level of TGF-β production in conditioned media after heat treatment was measured using ML-CCl64 cells, whose proliferation is specifically inhibited. At 3 and 7 days p.i. spleen cell cultures released TGF-β activity into the medium (Fig. 1c); part of the TGF-β was present in the active form (data not shown). The inhibitory TGF-β activity could be abolished by neutralizing antibodies against TGF-β₁ (Fig. 1c). The levels of TGF-β activity were not further increased in cultures stimulated with Con A (data not shown).

To evaluate directly TGF-β production in vivo, immunohistochemical studies were performed on splenic tissue sections prepared at 0, 5, 7 and 12 days p.i. Cells expressing TGF-β₁ protein were localized predominantly in red pulp regions of RCMV-infected rats at 5 and 7 days p.i., but not in uninfected rats or at 12 days after infection (data not shown). These results demonstrate the production of TGF-β₁ by splenocytes in the course of RCMV infection.

Detection of IL-1 production in RCMV-infected immunosuppressed rats

In contrast to the immunocompetent rats, radiation-immunosuppressed animals succumbed to an infection with...
was expressed as mean values ± SEM. The detection limit (DL) was 0.5 U/ml.

10^6 p.f.u. of RCMV within 8 to 10 days. No mortality was observed over a period of 30 days in PBS-injected animals after whole body irradiation only. At different time-points, sera from both groups were tested for the presence of IL-1 bioactivity. As depicted in Fig. 2(a), a peak of circulating IL-1 bioactivity (12 U/ml) was observed 1 day after irradiation. In vitro, infection of rat embryo fibroblasts resulted in the release of IL-1 activity comparable to that after LPS stimulation, whereas no activity was found in uninfected control cultures (Fig. 2b). Remarkably, at day 7 p.i., when rats had high concentrations of virus antigen in the spleen, liver, kidneys and lungs (Haagmans et al., 1994a), the sera of RCMV-infected rats were devoid of IL-1 bioactivity (< 0.5 U/ml, n = 8) while in the control rats values of 1.4 ± 0.4 U/ml (n = 6) were measured (Fig. 2a). In contrast, TNF-α levels gradually increased after RCMV infection but remained low in uninfected rats (Haagmans et al., 1994b). These data suggest the in vivo production of factor(s) which inhibit IL-1 bioactivity and/or its synthesis. Since TGF-β down-regulates IL-1 activity and induces IRAP (Turner et al., 1991; Wahl et al., 1993) we subsequently focused on TGF-β production.

**Inhibitory effect of sera from RCMV-infected immunosuppressed rats on the proliferation of T cells**

As depicted in Fig. 3, day 7 sera from RCMV-infected immunosuppressed rats inhibited the IL-2-induced proliferation of T cells. To identify the suppressive factor(s) present in infected rats, we tested for the presence of TGF-β. Inhibition of lymphocyte proliferation is a characteristic of TGF-β; when added as a recombinant protein this cytokine was inhibitory. Preincubation of the serum samples with anti-TGF-β antibodies restored the proliferative responses in the IL-2 assay (Fig. 3).

**Immunohistochemical localization of TGF-β in tissues of immunosuppressed RCMV-infected rats**

At day 7 p.i. immunohistological examination revealed high expression of RCMV antigen in the bone marrow, lungs, liver (Fig. 4a–c) and spleen. In the bone marrow, TGF-β1 expression was detected mainly in stromal cells (Fig. 4d), which also stained for RCMV antigen (Fig. 4a). In the lungs we observed an intense staining in the alveoli (Fig. 4e). Moreover, TGF-β1-producing cells were found in the liver (Fig. 4f). Some of these double-stained with an antibody against a 29 kDa cytoplasmic virus protein (Fig. 4g), while others remained positive only for TGF-β or virus antigen (Fig. 4g). Some infiltrating TGF-β1-positive lymphocytes were detected in the liver and lungs. At the antibody dilution used, staining in control rats was virtually absent; TGF-β1 expression was seen only in tissue underlying the epithelial lining of the conducting airways and large blood vessels, while the alveoli were negative (Fig. 4h). In the kidneys, which contained almost no virus antigen, no difference in TGF-β1 expression was noted between the groups. TGF-β2 expression was not enhanced in infected animals, except for the spleen (data not shown). Staining was absent in sections treated with preimmune serum (Fig. 4i).

**TGF-β activity after RCMV infection in vitro**

We then evaluated TGF-β production in RCMV-infected fibroblast cell cultures (> 95% of the cells were infected as determined by immunoperoxidase staining). Supernatants were harvested at 48 h p.i. and heated in order to activate TGF-β. RCMV-infected cells released TGF-β, which was neutralized...
by antibodies against TGF-β₁ (Fig. 5). The supernatants did not possess TGF-β activity prior to heat treatment. TGF-β production could be inhibited by preincubation of the virus inoculum with neutralizing hyperimmune RCMV serum but not with control sera (Fig. 5). We therefore conclude that RCMV is capable of inducing TGF-β upon infection of its target cells.

Discussion

As we have shown, CMV infection of immunocompetent or radiation-immunosuppressed rats leads to increased TGF-β expression. During RCMV infection, enhanced levels of TNF-α, but not IL-1 or IL-6, are found (Haagmans et al., 1994b, c). The local production of TGF-β, especially in the spleen, lungs and bone marrow stromal cells may contribute to the immunosuppression, interstitial pneumonia and inhibition of haematopoiesis observed in CMV disease.

TGF-β is a member of a superfamily of cytokines that includes TGF-α, activin and inhibin (for a review see Roberts & Sporn, 1990). It plays an important role in regulating repair and regeneration following tissue injury and in embryonic development. Since TGF-β is secreted by virtually all cell types in a biologically inactive form (Roberts & Sporn, 1990), its activity may be masked in vivo. Indeed, heat treatment of supernatants obtained from RCMV-infected cells resulted in the activation of TGF-β (Fig. 4). The mechanism of TGF-β activation in vivo is not exactly known, but conversion by plasmin or other macrophage-derived enzymes, which may be induced during the infection, has been postulated (Roberts & Sporn, 1990). Remarkably, inactive TGF-β administered to rats was converted into its active form, resulting in immunosuppression (Wallick et al., 1990). The ability of TGF-β to inhibit the proliferation of T and B cells and to suppress the activity of natural killer cells and macrophages at femtomolar concentrations (Kehrl et al., 1986; Tsunawaki et al., 1990) suggests a role in CMV-induced immunosuppression. Recently, it has been demonstrated that HCMV induces transcription and secretion of TGF-β₁ in fibroblasts (Michelson et al., 1994). The presence of RCMV antigen-positive cells that also express TGF-β suggests that, in vivo, at least a part of the TGF-β is induced directly by RCMV.

In addition to its effects on lymphocytes and monocytes, TGF-β also controls haematopoiesis, as shown in bone marrow culture systems (Keller et al., 1989). It suppresses the growth of immature haematopoietic cell populations while leaving more differentiated cells unaffected (Ishibashi et al., 1987). We observed that RCMV antigen and TGF-β were both localized in bone marrow stromal cells of RCMV-infected rats; TGF-β production may therefore impair recovery of immuno-suppressed rats from CMV infection. This is in concordance with observations made by Mutter et al. (1988), who concluded that it was not the CPE of murine CMV in host tissues but rather the failure of haematopoiesis that was the primary cause of death. In a subsequent study they showed that the in vitro generation of granulocyte/monocyte progenitors discontinued after infection of the stromal cell layer, whereas proliferation and differentiation remained unaffected (Busch et al., 1991). Human CMV can also impair haematopoiesis, either through infection of stromal cells and consequent perturbation of growth factor production or by direct infection of myeloid cells (Simmons et al., 1990).

The induction of TGF-β activity during RCMV infection may explain the absence of IL-1 bioactivity in the sera of radiation-immunosuppressed infected rats. After whole body irradiation, stimulation of myeloid growth factors including IL-1 is necessary to overcome the deleterious effects on bone marrow cells. Our observations confirm studies by Neta et al. (1991) who showed the importance of IL-1 in radiation protection. Our data further demonstrate that RCMV infection induces factors which suppress IL-1 bioactivity in vivo, despite its capacity to induce IL-1 expression in vitro (RCMV-infected fibroblasts are unable to convert latent TGF-β into the active form). These observations are in line with the human system, where IL-1 production but not its bioactivity is enhanced (Dudding & Garnett, 1987; Dudding et al., 1989). It remains to be determined whether TGF-β₁, which inhibits IL-1 activity and induces IRAP (Turner et al., 1991; Wahl et al., 1993), is responsible for this suppression. Inhibition of IL-1 activity during the infection may prevent normal immune responses and impair haematopoietic repopulation of the bone marrow after whole body irradiation.

Immunohistochemical staining and in situ hybridization demonstrated TGF-β expression in normal adult mouse lung

![Graph](image-url)
Fig. 4. For legend see facing page.
tissue, notably the tissue underlying the epithelial lining of the conducting airways and large blood vessels (Flanders et al., 1989). In the RCMV-infected rats we observed intense staining not only in these tissues but also in the alveoli. The induced TGF-β may initiate a complex sequence of events, including chemotraction of monocytes and leukocytes, induction of angiogenesis and control of the production of cytokines and other inflammatory mediators (Roberts et al., 1986). The ability of TGF-β to regulate extracellular matrix deposition may thus partly result in the pneumonia observed in CMV disease; TGF-β stimulates the synthesis of matrix components including fibronectin, tenascin, collagens and proteoglycans (Laiho et al., 1989). Simultaneously it blocks matrix degradation by decreasing the synthesis of proteases and increasing the levels of protease inhibitors (Roberts et al., 1986).

Both suppressive and inductive effects on human immuno-deficiency virus expression result from TGF-β stimulation of monocytic and T lymphocytic cells (Poli et al., 1992), and an important role in the immunodeficiency infection has been postulated for TGF-β (Kekow et al., 1990; Wahl et al., 1991). Alcami et al. (1993) reported enhanced replication of human CMV upon treatment with TGF-β. We have seen similar effects on RCMV replication in macrophages (B. L. Haagmans, K. J. Teerds, A. J. M. van den Eijnden-van Raaij, M. C. Horzinek & V. E. C. J. Schijns, unpublished results). From all these observations we are led to postulate that TGF-β may play an important role in the pathogenesis of CMV disease by enhancing virus replication, inducing immunosuppression and pathology.

We thank Dr Kathy Flanders (National Cancer Institute, NIH, Bethesda, Md., USA) for providing anti-TGF-β, antibodies. André M. M. Millenburg for the IL-1 assay and Dr P. van der Meide (TNO, Rijswijk, The Netherlands) for the DB-1 and DB-12 antibodies. This work was supported by grants from TNO Rijswijk (B.L.H.) and the Royal Netherlands Academy of Arts and Sciences (K.J.T.).

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Received 8 May 1996; Accepted 17 September 1996